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Martina Flynn
Licensing Associate
Office of Technology Transfer
Dana Farber Cancer Institute
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Dear Martina:

Following our communication I am returning the completed Invention Disclosure forms, on AMPLIFICATION OF DNA IN A HAIRPIN STRUCTURE, AND APPLICATIONS.

Thank you.

Sincerely

G. M. Makrigiorgos, Ph.D

PCR-based amplification is used in almost every aspect of genetic diagnosis, DNA cloning, mutation detection and basic research. A major problem with PCR, however, is that all polymerases invariably generate errors during amplification. Polymerase misincorporations lower the quality of DNA cloning or protein functional analysis by in vitro translation and set a limit for molecular mutation detection methods of 1 mutant sequence in a background of 10^5 - 10^6 wild type sequences. Thus high selectivity mutation detection, which relies on PCR, falls short by 1-2 orders of magnitude of the selectivity required to investigate mechanisms of spontaneous mutagenesis, to identify cancer cells at an early stage, to detect minimal residual disease, or to detect mutations in single cells. In all these applications, polymerase misincorporations invariably become disguised as mutations and result to false positives. Here we present hairpin PCR, a new form of PCR which completely separates genuine mutations from polymerase misincorporations to allow the generation of 'error free' amplified DNA for mutation detection or other applications. Hairpin PCR operates by converting a DNA sequence to a hairpin following ligation of oligonucleotide caps to top and bottom DNA strands. We show that during DNA synthesis, the polymerase copies both DNA strands in a single pass. Forcing the enzyme to keep a double record of the sequence effectively boosts the DNA replication fidelity, as it is unlikely that a misincorporation will happen at the same position in both DNA strands simultaneously. In hairpin PCR, when a misincorporation occurs it automatically forms a 'mismatch' following DNA amplification, and is distinguished from genuine mutations that remain 'fully matched'. Error-free DNA can subsequently be isolated from mismatch-containing DNA using one of many approaches, such as dHPLC, CDCE or enzymatic depletion. We present feasibility for the main technical steps involved in this procedure, conversion of a sequence to a hairpin that can be PCR-amplified, exponential amplification from picogram amounts, conversion of base misincorporations to mismatches and separation of mismatches from homoduplex DNA using dHPLC.

The present hairpin-PCR establishes a strategy for almost complete elimination of PCR errors that can be expected to allow a major boost to the detection of mutations in human tissue. The new amplification of DNA in a hairpin structure should also be applicable in the field of molecular beacons and quantitative real time PCR.

COPY

* Please type or print * Use Additional paper if needed; initial each page	**DANA FARBER CANCER INSTITUTE INVENTION DISCLOSURE**	DFCI NO. (OTT USE ONLY) 841 OTHER REFERENCES (OTT USE ONLY)

1. TITLE OF INVENTION

AMPLIFICATION OF DNA IN A HAIRPIN STRUCTURE, AND APPLICATIONS

2. PARTICIPANTS - Please list all individuals who were associated with making the invention including all DFCI investigators, post-doctoral fellows, technicians and non-DFCI collaborators

NAME	HOME ADDRESS	CITIZEN-SHIP	DEPARTMENT/ INSTITUTION
G. M. Makrigiorgos, Ph.D tel: (617) 632-6905 (work) 734-4656 (home)	63 Payson Road Brookline MA 02467	Greek	Radiation Oncology/DFCI

3. SOURCES OF FUNDING - Identify all sponsors of the work that led to the Invention

SPONSOR (Federal or Commercial)	PRINCIPAL INVESTIGATOR
AJCRT FOUNDATION	**Departmental funds**	**G.M. Makrigiorgos, Ph.D**

4. DISCLOSURE HISTORY	DATE	LOCATION	REFERENCES AND COMMENTS (USE SEPARATE SHEET IF NECESSARY)
A. Invention development records (e.g. notes, drawings, reagent orders, notebooks, computer records, etc.)	January 2002	PI's Office And lab note - books	De-novo procedure to amplify DNA in hairpin structure and its application in generating DNA without PCR errors was confidentially disclosed to close collaborator.
B. First publication containing a description of the invention (published, submitted or ms. in preparation)	None		none
C. Other External disclosures (e.g., seminars, posters, verbal disclosures outside DFCI)	None		Note: the invention was verbally disclosed (confidentially) to close collaborators.

5. LIST MATERIALS ASSOCIATED WITH INVENTION DISTRIBUTED TO INDIVIDUALS OUTSIDE OF DFCI

Material Designation	Recipient	Institution
None		

6. LIST MATERIALS OBTAINED FROM OUTSIDE SOURCES AND USED IN INVENTION

Material Designation	Provider	Institution
None		

PARTICIPANTS(as listed In 2., above): PLEASE SIGN INITIALS HERE

7. A. GENERAL DESCRIPTION OF INVENTION (identify, date and list attachments describing invention)

BACKGROUND

See attached description

THE CURRENT INVENTION
See attached description

ATTACHED MATERIALS: detailed description.

POSSIBLE USES OF THE INVENTION

See attached description

See also attached description

C. IDENTIFY THE NAMES OF CLOSEST SCIENTIFIC PEERS IN THE FIELD OF THE INVENTION

8. **INVENTION SUBMITTED BY**		**WITNESSED BY:** (A person who is not a participant and has read and understood the disclosure)
(PARTICIPANT'S SIGNATURE)	**(DATE)**	(SIGNATURE OF WITNESS) (DATE) **(LEAVE BLANK)**
(PARTICIPANT'S SIGNATURE)	**(DATE)**	
(PARTICIPANT'S SIGNATURE)	**(DATE)**	
(PARTICIPANT'S SIGNATURE)	**(DATE)**	

AMPLIFICATION OF DNA IN A HAIRPIN STRUCTURE, AND APPLICATIONS

-confidential

-written by: G. Mike Makrigiorgos, Ph.D

1. BACKGROUND

The ability to perform mutation detection with high selectivity would have a major impact in cancer research and diagnosis. Scanning for very low frequency mutations occurring naturally in somatic tissues (< 1 mutant in 10^7 alleles) or at early stages of carcinogenesis would allow a better understanding of the origins of genomic instability and the identification of tumor signatures as markers for early tumor detection. Identification of mutations in somatic tissues following environmental exposures would enable generation of carcinogen-specific mutational fingerprints [1]. Reliable screening for 'onco-mutations'[2, 3] would enhance the clinical and diagnostic utility of minimal residual disease detection and identification of mutations in bodily excretions [4].

Most mutation detection technologies however fall short of the enormous sensitivity needed to address these issues. The most selective technologies invariably rely on PCR, but PCR also poses a final sensitivity limit, typically 1 mutant in 10^2 – 10^6 alleles, since all DNA polymerases generate errors during DNA synthesis which can be misinterpreted as mutations (false positives). Both unknown and known mutation detection methods are affected by PCR errors and the most sensitive methods are those that are affected most. The sensitivity of constant denaturant capillary electrophoresis (CDCE), a powerful method for unknown mutation scanning, is limited by the fidelity of Pfu polymerase [1]. Some of the highest sensitivity assays for known mutation detection (PCR/RE/LCR [5]; MutEx-ACB-PCR [6]; Radioactivity-based PCR-RFLP [7]; RSM (Ref); APRIL [8] and others reviewed in [9]) utilize PCR in at least one stage prior to selection (RFLP), and are therefore also limited by PCR errors [3]. In summary, the ability to amplify DNA without being limited by polymerase-introduced errors would have a very substantial impact in mutation detection and in cancer diagnosis.

We have invented hairpin-PCR, a novel method that allows elimination of PCR errors in any sequence of interest, and which would supply several existing technologies with the needed 'sensitivity leap'. By converting the sequence of interest into a hairpin and performing PCR in a hairpin-structure, polymerase-generated single nucleotide changes, small insertions and deletions can be separated from true mutations, thereby providing practically error-less DNA.

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2. DESCRIPTION OF THE INVENTION

In this approach, a DNA sequence which needs to be PCR-amplified is first converted to a hairpin following ligation of an oligonucleotide 'cap' on one end and a pair of non-complementary linkers on the other end (**Figure 1**). Next, primers corresponding to the two non-complementary linkers are used in a PCR reaction that proceeds by displacing the opposite strand and amplifying the entire complement of the original hairpin. We discovered that, by following this strategy, exponential PCR amplification of the hairpin is enabled with an efficiency similar to that of regular PCR (see Section 3). Following hairpin amplification, the PCR product is purified, then heat-denatured to allow the hairpins to separate from their complementary strand, and placed rapidly on ice. Because of the sudden cooling,

cross-hybridization of different hairpins is minimal, and thus the original hairpins are re-formed, following their amplification.

By amplifying DNA in a hairpin-formation, polymerase-errors practically always end-up forming a mismatch. Genuine mutations, however, remain fully-matched. For example, if the polymerase introduces an A>G mutation on the upper strand of the original sequence, it is very unlikely that, during synthesis of the bottom strand of a single hairpin it will perform the *exact opposite* error (T>C mutation) at exactly the same position. Even for a polymerase with a large error rate of 10^{-4}/base the odds for a double-error event are $10^{-4} \times 10^{-4} \times 0.25 = 2.5 \times 10^{-9}$, i.e. less than the expected spontaneous mutation rate in somatic tissues [10, 11]. On the other hand, practically all genuine mutations remain fully matched following hairpin-PCR, as these reside on both strands from the beginning (**Figure 1**). Next, the amplified hairpins that contain mismatches are efficiently separated from those that do not, using dHPLC-mediated fraction collection (see Section 3). With subsequent removal of the hairpin caps, the error-free amplified DNA can be processed for mutation detection without being limited by polymerase errors.

Both DGGE and dHPLC, as well as the mismatch-binding protein MutS–based methods had previously been used to separate the fraction of PCR-amplified sequences containing mismatches [12, 13, 14, 15]. All of these methods, however, utilize the conversion of PCR errors to mismatches via cross-hybridization of amplified products, and in this process PCR errors are also inadvertently converted to mismatches. When mutations are at a low frequency, practically all of them will be converted to mismatches, thus there is no way to discriminate them from PCR errors. Thus there is no benefit for mutation detection.

Hairpin PCR takes advantage of the fact that, genuine mutations are witnessed in both upper and lower DNA strands while PCR errors occur only on one strand at a time. By forcing DNA polymerase to perform 'double duty', (i.e. to copy both the upper and the lower DNA strands in a single pass), hairpin PCR automatically converts PCR errors -but not mutations- to mismatches. This approach allows to efficiently (>100-fold) separate mismatches from mutations and practically eliminates the polymerase-generated single nucleotide changes, small insertions and deletions.

3. ENABLEMENT OF THE INVENTION

Hairpin-PCR. PCR-amplification of a hairpin has traditionally been considered an inefficient process [16]. Several methods utilize hairpin formation as a way to abolish PCR amplification (e.g. 'suppression subtractive hybridization' [16]). We hypothesized that the main reason PCR is abolished by hairpins is not polymerase-related, but the inability of PCR primers to bind to their respective binding sites at the end of the hairpin molecules (e.g. **Figure 2, hairpin A**). To test this hypothesis, we designed hairpins with non-complementary ends (**Figure 2, hairpins B-C**) which do not inhibit primer binding. Hairpins A-C were synthesized by Oligos Etc. (Oregon, USA). 1 ng each hairpin was then used in a 25 µl PCR reaction using Titanium Advantage^{R} polymerase (Clontech) and the primers 5'-GTG AGA GGC TGG AGA GTG CT-3', forward; and 5'-ACG TCG ACT ATC CGG GAA CA-3', reverse. PCR thermo-cycling conditions were: 94°, 30 sec; (94°, 30 sec / 68°, 60 sec) x 25 cycles; 68°, 60 sec; 4°; Hold. Using the same thermocycling conditions, quantitative real time PCR in the presence of SYBR Green I dye was also performed in a Cepheid I machine available in our lab.

It can be seen (**Figure 3A**) that hairpins B-D produce the expected PCR product, while hairpin A does not. Omission of either the forward or the reverse primers abolishes the product which indicates that the full length hairpin is replicated by the polymerase, and that the amplification is exponential and

requires both primers. Because of the way hairpin-PCR operates (**Figure 1**), the resulting PCR products are expected to be double-stranded DNA, each strand of which is a full hairpin. To separate the two strands, and to recover the original hairpins, following purification of the PCR product (QIAquick™ centrifugation columns, Qiagen Inc) the samples were denatured at 95° C, 1 min, and suddenly cooled by placing them directly on ice. This procedure does not allow time for substantial cross-hybridization of different DNA strands, while each strand is expected to rapidly form a hairpin due to its self-complementary sequence. **Figure 3B** demonstrates that this process converts the product of hairpin C amplification to a band half the size, which corresponds to monomer hairpin C

Sequence-specific PCR amplification of a hairpin. As demonstrated in **Figure 3,** DNA hairpins can readily be PCR-amplified as long as primers can bind to their two ends where generic non-complementary linkers have been ligated. To demonstrate that the process can also be sequence specific, we synthesized primers which bind to the generic linkers but also extend into the sequence of interest. Either a single primer, or both forward and reverse primers were made to extend into the hairpin C sequence. **Figure 4** demonstrates amplification of hairpin C using primers that extend 0, or 12 nucleotides into the hairpin sequence, while they *still* overlap the linker sequence (lanes 1 and 2 respectively). When 20-mer primers *fully* extending into the hairpin-forming p53 sequence and not overlapping the linker sequence are used, no hairpin-PCR product is obtained (lane 3). These data are consistent with the hypothesis that, as long as primer binding occurs, polymerase effectively displaces the opposite strand and polymerizes the hairpin. Consistent also with published observations, if the full primer length is included in the hairpin-forming sequence no amplification occurs.

Efficiency of hairpin-PCR. To investigate whether hairpin PCR has the sensitivity needed to amplify single-copy sequences from genomic DNA, serial dilutions of hairpin D into human genomic DNA were used. Purified genomic DNA from the HL60 cell line that lacks the p53 gene was purchased from the American Type Culture Collection, ATCC (Manassas, VA). Each hairpin-PCR reaction contained 100 ng genomic DNA plus decreasing amounts of hairpin B spiked in the reaction. 1 human genome (~$3x10^9$ bp) is about $2.7x10^7$ times the size of 1 hairpin B. Since there are two copies of each sequence in the genome, it can be estimated that spiking of 10^{-5} ng hairpin B into 100 ng HL60 DNA is approximately equivalent to having a single copy gene in a hairpin formation in the genome. **Figure 5** demonstrates application of hairpin PCR using the PCR primers and conditions described above. It can be seen that hairpin PCR can readily amplify targets from genomic DNA, i.e. it is of similar efficiency as regular PCR.

Separation of mismatched vs. fully-matched hairpins, via dHPLC. In order to obtain the fraction of hairpin-amplified DNA that does not contain PCR errors, we need to eliminate the mismatch-containing hairpins, as per **Figure 1**. dHPLC chromatography is an established technique for distinguishing heteroduplexes from homoduplexes [17, 18, 19]. In this approach, DNA containing single base pair mismatches is retained for shorter times on the dHPLC column than homoduplex DNA at specific temperatures, and thereby the two can be separated and collected in separate containers via a fraction collector. Such dHPLC separation is well established for sequences up to about 450 bp long, although separations for sequences up to 1500 bp have also been reported [20]. Following PCR amplification PCR errors can be converted to mismatches via cross-hybridization and distinguished from homoduplex, error-free DNA (**Figures 6 and 7**, data from Transgenomics Inc, Cambridge, MA – [21, 22]).

However, for the purposes of mutation detection, neither dHPLC nor other methods can be used to eliminate PCR errors, because low-frequency mutations will also be converted to mismatches and

will be lost in this process. On the other hand hairpin PCR retains the mutations within the homoduplex peak while PCR errors are still expected to fall within the heteroduplex peak, as explained in section B1.

To demonstrate that PCR-amplified hairpins containing a single base mismatch (such as those expected to result from a polymerase misincorporation) can be distinguished from fully-matched hairpins via dHPLC, we applied hairpin-PCR to hairpin B (see Figure 2). At the same time, two more hairpins, B1 and B2, were synthesized and PCR-amplified. These were identical in sequence as hairpin B, except that they were deliberately synthesized to contain a single base change at position 47 (47G>A, hairpin B1) or a 3 base insertion at position 46 (46insACA, hairpin B2). Therefore hairpins B1 and B2 simulate a polymerase single base mis-incorporation or a three base 'slippage error', respectively. 100 ng of the PCR products were then heat-denatured and rapidly cooled on ice so that each strand folds into an individual hairpin, as described above. The amplified hairpins were then run on a Transgenomics dHPLC WAVE system at the company's headquarters (Cambridge, MA). **Figures 8 and 9** demonstrate that hairpins B1 and B2 could be clearly distinguished from the fully-matched hairpin B at a partially denaturing temperature of 61°C. Therefore the homoduplex, error-free DNA can be separated from the heteroduplex, error-containing DNA, and collected in a dHPLC fraction collector.

Conversion of a given sequence to a hairpin via ligation.

The results in **Figures 2-9** were obtained using sequences synthesized directly in the form of a hairpin, via an oligonucleotide synthesizer. In practice, however, one needs to start from normal double stranded DNA and convert it to a hairpin, prior to performing hairpin-PCR. For a given sequence, this is performed by a single ligation step, during which a hairpin 'cap' is added on one end of the sequence, and two non-complementary linkers are added on the other end. Ligation of oligonucleotide caps are common in the literature and should not present a problem [23]. On the other hand ligation of non-complementary linkers can be performed by ligation of an oligonucleotide cap, which has a centrally-located uracil. Following ligation of the cap to the sequence of interest, uracil glycosylase is utilized to generate an abasic site, following by heating which converts the abasic site to a strand break. As a result, two non-complementary ends are generated at the end of the sequence. An example of such a uracil-containing sequence is the following sequence:

5'GGCGCGCCGATGAAGGCAGCTGACCCCATCTCATUUCTTCTCCCCCTCCTCTGTTGCTCA TCGGCGCGCCCATG3'where UU are two consecutive uracils. The above structure is a hairpin cap which can ligate to any position on a given sequence which has been digested by the NIa-III restriction endonuclease enzyme.

To convert a given sequence within genomic DNA to a hairpin, the genome is cut with two enzymes, e.g. NIa-III and TaqI, to generate sequences that contain the NIa-III recognition site on one end, and the TaqI site on the other. Hairpin-caps are then simultaneously ligated on the two sites. The hairpin cap containing the uracil is then converted to the non-complementary linker part of the hairpin. Sequence-specific primers overlapping the non-complementary linkers as well as the actual sequence of interest are used to PCR-amplify the sequence of interest from genomic DNA in a hairpin-structure. This is then processed for dHPLC-separation of the DNA which contains PCR errors. If needed, the dHPLC injection can be repeated more than once to achieve better separation of homoduplexes and heteroduplexes. The error-free homoduplexes can then be used for ultra-sensitive mutation detection, without being limited by polymerase errors.

4. CONCLUSION

PCR-induced errors have been regarded as an 'ultimate frontier' for mutation detection. By providing error-less amplified DNA for analysis, the present hairpin PCR will allow a major boost to almost every existing or future method for highly selective mutation detection and enable studies and diagnostic tests that were impossible with previous technology. Furthermore, the ability to amplify DNA hairpins is new to the area of molecular biology, and it is expected to find additional applications, such as in the diagnostic uses of molecular beacons, and others.

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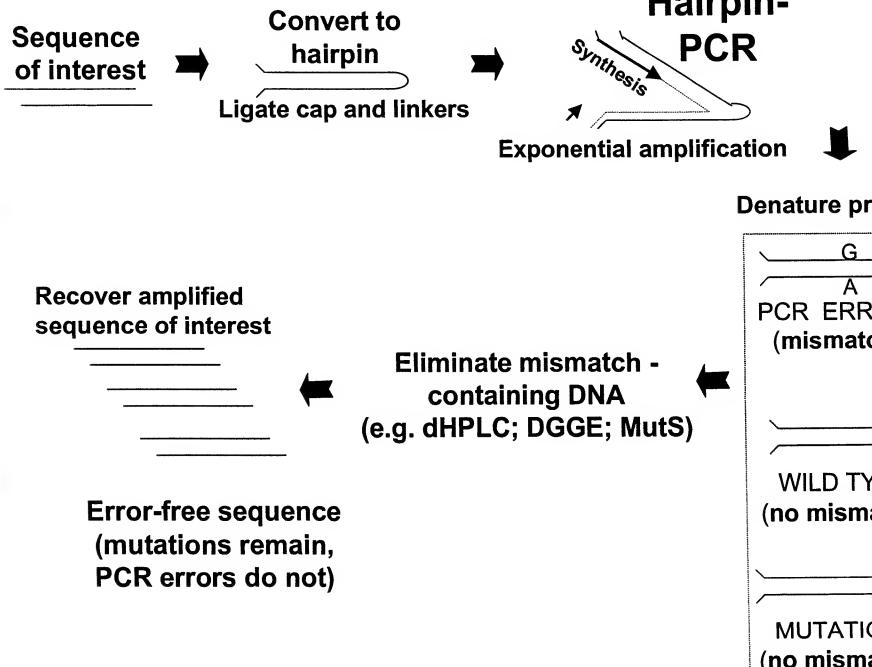

Sequence of interest
Convert to hairpin
Ligate cap and linkers
Hairpin-PCR
Synthesis
Exponential amplification
Denature products
G
A
PCR ERROR (mismatch)
G
C
WILD TYPE (no mismatch)
A
T
MUTATION (no mismatch)
Eliminate mismatch - containing DNA (e.g. dHPLC; DGGE; MutS)
Recover amplified sequence of interest
Error-free sequence (mutations remain, PCR errors do not)

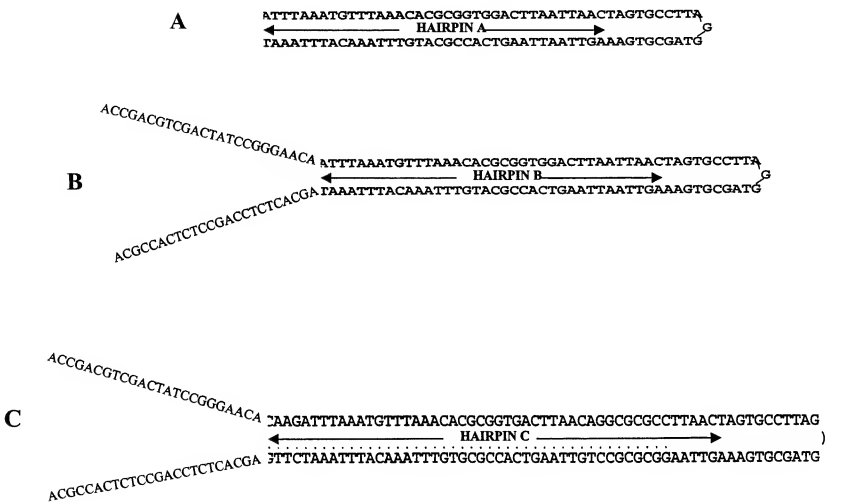
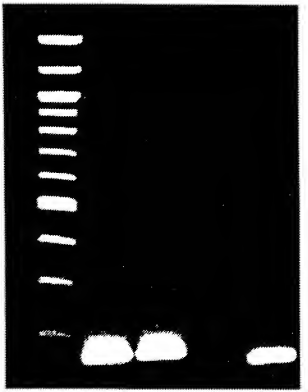
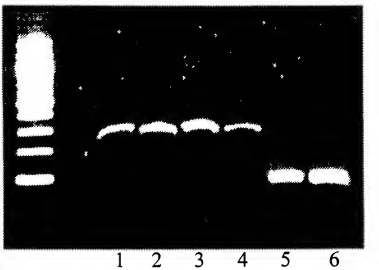

A
ATTTAAATGTTTAAACACGCGGTGGACTTAATTAACTAGTGCCTTA
HAIRPIN A
TAAATTTACAAATTTGTACGCCACTGAATTAATTGAAAGTGCGATG
B
ACCGACGTCGACTATCCGGGAACA
ATTTAAATGTTTAAACACGCGGTGGACTTAATTAACTAGTGCCTTA
HAIRPIN B
TAAATTTACAAATTTGTACGCCACTGAATTAATTGAAAGTGCGATG
ACGCCACTCTCCGACCTCTCACGA
C
ACCGACGTCGACTATCCGGGAACA
AAGATTTAAATGTTTAAACACGCGGTGACTTAACAGGCGCGCCTTAACTAGTGCCTTAG
HAIRPIN C
TTCTAAATTTACAAATTTGTGCGCCACTGAATTGTCCGCGCGGAATTGAAAGTGCGATG
ACGCCACTCTCCGACCTCTCACGA

Figure 2

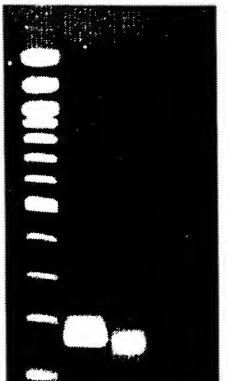


A. Lanes 1-4, hairpin PCR of hairpin C, hairpin C (duplicate), hairpin A, and hairpin B, respectively



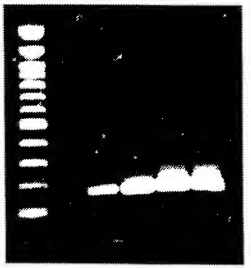
B. Lanes 1-4, hairpin PCR of hairpin C (4 replicates). Lanes 5-6, the same PCR products following denaturation at 95C and rapid placing on ice (original hairpin is reformed).

FIGURE 3



Sequence-specific hairpin PCR.
Lanes 1-3, hairpin PCR of hairpin C using primers that do not overlap the hairpin portion (lane 1), or overlapping by 12 nucleotides the hairpin portion (lane 2), or fully resting inside the hairpin portion (lane 3).

Figure 4



0 1pg 10pg 0.1ng 1ng

Hairpin PCR performed following spiking of decreasing amounts hairpin B into 100 ng genomic DNA. The amount hairpin B spiked into the human genome is depicted.

Figure 5

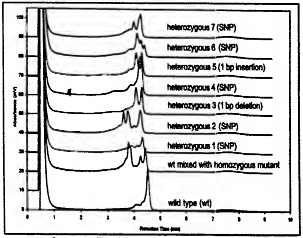


Figure 6: Partially-denaturing WAVE® System detection (71°C) of 8 different polymorphisms in a 269 bp fragment.

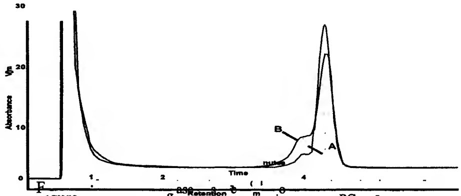
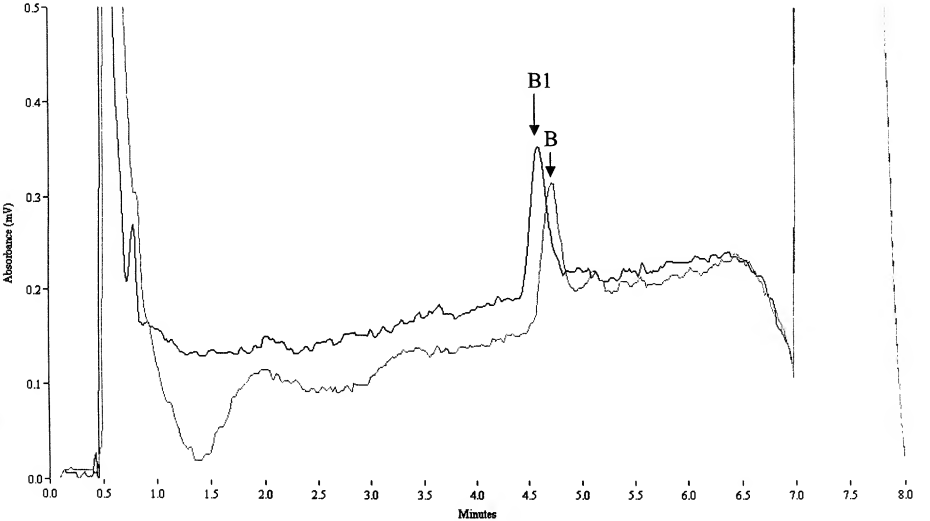
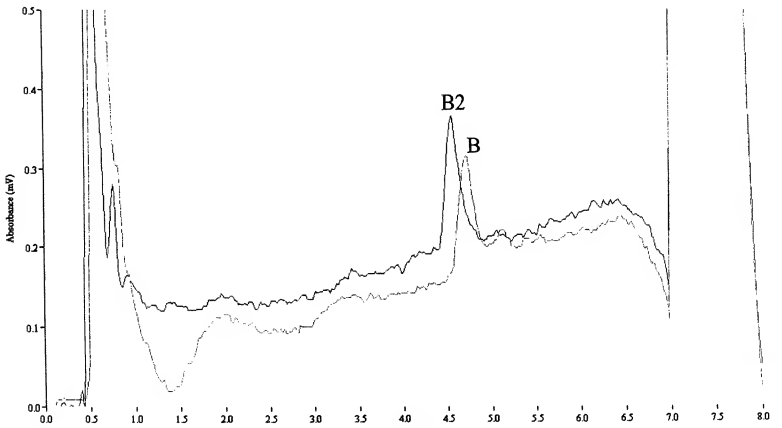


Figure . The effe o g in o ps. PC R ... Th effe mi rati durin R o pe files obtain us g the ... eras ... the Optimase ... A) ... bump ... the wel ... d fin m pe ... ve ... on ... PC ... ed ... ak ... eras (B ... o few s co orati d during R. An a tern tive lym ...) shows a distinct 'shoulder' indicating a higher level of misincorporation.



Separation of PCR-amplified hairpin B (wild type) and B1 (containing a mismatch), using dHPLC.

Figure 8



Separation of PCR-amplified hairpin B (wild type) and B2 (containing a 3 base insertion), using dHPLC.

Figure 9

NOVEL AMPLIFICATION OF DNA IN A HAIRPIN STRUCTURE: TOWARDS A RADICAL REDUCTION OF PCR ERRORS FROM AMPLIFIED DNA

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PCR-based amplification is used in almost every aspect of genetic diagnosis, DNA cloning, mutation detection and basic research. A major problem with PCR, however, is that all polymerases invariably generate errors during amplification [1-4]. Polymerase misincorporations lower the quality of DNA cloning [5] or protein functional analysis by in vitro translation [6] and set a limit for molecular mutation detection methods of 1 mutant sequence in a background of 10^5 - 10^6 wild type sequences [7, 8]. Thus high selectivity mutation detection, which relies on PCR, falls short by 1-2 orders of magnitude of the selectivity required to investigate mechanisms of spontaneous mutagenesis [9], to identify cancer cells at an early stage [10], to detect minimal residual disease [11], or to detect mutations in single cells [6]. In all these applications, polymerase misincorporations invariably become disguised as mutations and result to false positives [12]. Here we present hairpin PCR, a new form of PCR which completely separates genuine mutations from polymerase misincorporations to allow the generation of 'error free' amplified DNA for mutation detection or other applications. Hairpin PCR operates by converting a DNA sequence to a hairpin following ligation of oligonucleotide caps to top and bottom DNA strands. We show that, unlike regular DNA hairpins, a DNA hairpin with non-complementary ends can be PCR-amplified and that during DNA synthesis, the polymerase copies both DNA strands in a single pass. Forcing the enzyme to perform this double duty effectively boosts the DNA replication fidelity, as it is unlikely that a misincorporation will happen at the same position in both DNA strands simultaneously. In hairpin PCR, when a misincorporation occurs it automatically forms a 'mismatch' following DNA amplification, and is distinguished from genuine mutations that remain 'fully matched'. Error-free DNA can subsequently be isolated from mismatch-containing DNA using one of many approaches, such as dHPLC, CDCE or enzymatic degradation. We present feasibility for the main technical steps involved in this strategy, conversion of a sequence to a hairpin that can be PCR-amplified, exponential amplification from picogram amounts, conversion of base misincorporations to mismatches and separation of mismatches from homoduplex DNA using dHPLC.

The present hairpin-PCR establishes a strategy for almost complete elimination of PCR errors that can be expected to allow a major boost to the detection of mutations in human tissue. The new amplification of DNA in a hairpin structure is expected to also have applications in the field of molecular beacons [13, 14] and quantitative real time PCR.

Results and Discussion.

Hairpin PCR is based on the novel observation that, unlike regular DNA hairpins that suppress PCR[15], a DNA hairpin with non-complementary ends can readily be PCR-amplified. For this purpose, a DNA sequence which needs to be PCR-amplified is first converted to a hairpin following ligation of an oligonucleotide 'cap' on one end and a pair of non-complementary linkers on the other end (**Figure 1**). Primers corresponding to the two non-complementary linkers are then used in a PCR reaction that proceeds by displacing the opposite strand and amplifying the entire complement of the hairpin. The primers can optionally overlap the sequence of interest to confer sequence specificity. Following hairpin amplification, the PCR product is heat-denatured to allow the hairpins to separate from their complementary strand, and placed rapidly on ice. Because of the sudden cooling, cross-hybridization of different hairpins is minimal, and thus the original

hairpins are re-formed, following their amplification. By amplifying DNA in a hairpin-formation, polymerase-errors should practically always end-up forming a mismatch. If the polymerase introduces an A>G mutation on the upper DNA strand it is unlikely that, during synthesis of the bottom strand of a single hairpin it will perform the exact opposite error (T>C mutation) at the complementary-strand position. Even for a polymerase with a large error rate of 10^{-4}/base the odds for a double-error event are $10^{-4} \times 10^{-4} \times 0.25 = 2.5 \times 10^{-9}$, i.e. less than the expected spontaneous mutation rate in somatic tissues [16, 17]. On the other hand, practically all genuine mutations remain fully matched following hairpin-PCR, as these reside in both strands from the beginning (**Figure 1A**). This complete discrimination of polymerase errors from the mutations allows subsequent isolation of error-free amplified hairpins by one of many strategies, such as via dHPLC or CDCE-mediated separation or via enzymatic elimination of mismatches. By removing the hairpin caps from the error-free hairpins, the original sequence is recovered following its amplification.

To test our hypotheses, we designed long oligonucleotides (B,C,D,E, 149, 168, 200, and 218 nucleotides respectively) expected to form hairpins with non-complementary ends which do not inhibit primer binding at their ends (**Figure 1B**), as well as a regular hairpin A, 150 bp, which lacks the non-complementary ends. Hairpins D and E encompass the complete sequence of p53 exon 9. 1 ng each hairpin was then used in a 25 µl PCR reaction using Titanium Advantage^{R} polymerase (Clontech) and primers designed to operate on the non-complementary ends of hairpins B-D, or alternatively on the complementary ends of hairpin A. Hairpins B-D produce a PCR product, while hairpin A does not (**Figure 1C, lanes 1-5**). Hairpins are readily amplified as long as primers are allowed to bind, and the polymerase is able to synthesize the hairpin complement, presumably by displacing the opposite strand. Omission of either forward or reverse primers abolishes the product (**Figure 1C, lanes 6-7**) which indicates that amplification requires both primers and that the full length hairpin is replicated by the polymerase. Hairpin PCR was repeated using two proof-reading polymerases, Pfu Turbo^{TM} (Stratagene), or Advantage-HF2 (Clontech) and amplification was obtained (**Figure 1D**). **Figure 4B** depicts quantitative real-time hairpin-PCR profiles of hairpin D serial dilutions, using SYBR Green I dye. The exponential nature of amplification is evident. Because of the way hairpin-PCR operates (**Figure 1A**), the PCR products are expected to result to double-stranded DNA molecules, each strand of which is a full hairpin. To separate the two strands, and to recover the original hairpins, following purification of the PCR product the samples are denatured at 95° C, 1 min, and rapidly cooled by placing them directly on ice. This procedure does not allow time for substantial cross-hybridization of different DNA strands, while each strand is expected to rapidly form a hairpin due to its self-complementary sequence. **Figure 1E** demonstrates that rapid cooling converts the hairpin amplification product (**lanes 1 and 2**) to a band approximately half the size (**lanes 5 and 6**), which corresponds to the expected monomer hairpin. Next, the forward and reverse primers used for the amplifications in **Figure 1B** were re-designed to encompass an additional 9 nucleotide extension (20+9=29mers) inside the p53 exon 9 hairpin D sequence. **Figure 1F** demonstrates that, although the 3'-end of the primers falls within the hairpin portion of the sequence, amplification remains almost unhindered. The data are consistent with the occurrence of primer binding, due to the 20 bases overlap with the non-complementary end of the hairpin, and that the 3'end

of the primers temporarily displaces the hairpin portion. This 'invasion' at the DNA ends by hybridized oligonucleotides[18] presumably happens frequently enough to allow polymerase binding and primer extension to occur. Therefore restricting the primers on the non-complementary ends amplifies every hairpin sequence that contain those ends, while by utilizing primers whose 3' ends extend into the hairpin sequence hairpin-PCR is rendered sequence specific.

To investigate whether hairpin PCR has the amplification efficiency needed to amplify single-copy sequences from genomic DNA, serial dilutions of the p53 exon 9-containing hairpin D into human genomic DNA were used. 100 ng purified genomic DNA from a cell line that lacks the p53 gene (ATCC CRL-1543) was mixed with decreasing amounts of hairpin D. One human genome (~$3x10^9$ bp), is ~$1.5x10^7$ times the size of hairpin D therefore spiking 10^{-2} pg hairpin D into 100 ng genomic DNA is approximately equivalent to adding a single copy p53 exon 9 in a hairpin formation in the genome. **Figure 2A** and **B** demonstrate hairpin PCR amplification of p53 exon 9 using two different polymerases. Amplification from 0.01-0.1 pg hairpin D in the presence of complex genomic DNA is obtained. The amplification efficiency of hairpin PCR appears comparable to that of regular PCR.

To demonstrate that PCR-amplified hairpins containing a single base mismatch, such as those expected to result from polymerase misincorporations, can be distinguished from fully-matched hairpins via dHPLC, we injected homoduplex PCR-amplified hairpins into a WAVE™ dHPLC system (Transgenomics Inc) equipped with a fraction collector. Two more hairpins were synthesized and PCR-amplified. These were identical to the homoduplex hairpin except that they were synthesized to contain base changes 47G>A and 45insACA, which represent a common misincorporation by Taq polymerase and a slippage error, respectively [19]. PCR products were then heat-denatured and rapidly cooled, so that each strand folds to an individual hairpin and the base substitutions in the mutant hairpins are expected to form mismatches (heteroduplex hairpins). 1 ng each heteroduplex and homoduplex hairpin was injected separately into dHPLC, or, alternatively, mixed (1:1) and injected as a mixture. At a partially denaturing temperature of 61°C, the peaks from the heteroduplex hairpins could be distinguished from the fully-matched, homoduplex hairpin, **Figure 2B**. Setting the threshold of the fraction collector on the trailing part of the homoduplex peak allows the collection of mainly (70-80%) homoduplex hairpin out of this mixture. This example simulated a worse case scenario, where the heteroduplex DNA was 50% of the overall sample. Normally however, the heteroduplex peak resulting from PCR errors will be a smaller fraction (~1-10%) of the homoduplex peak [2]. From the data in **Figure 2B** it can be estimated that if PCR errors are confined to 10% or 1% of the sequences, one would collect >95% and >99% homoduplex DNA respectively, resulting to a radical elimination of heteroduplex hairpins from the mixtures. In dHPLC all possible base changes and PCR errors are detectable[20], however individual base changes can result to varying degrees of separation of heteroduplexes from the homoduplex peak [21]. Nevertheless, collection of the trailing portion of the homoduplex will always filter-out the misincorporations. Further elimination of heteroduplexes should be possible by re-cycling the collected homoduplex through the dHPLC, for a second time.

To enable the scheme in **Figure 1A,** conversion of a native DNA fragment to a hairpin that can be amplified is required. To convert a sequence to a hairpin with non-

complementary ends we performed ligation of two different oligonucleotide 'caps', Cap1 and Cap2, at the positions of two restriction sites encompassing the sequence (**Figure 2C**). Cap1 and Cap2 are small oligonucleotides designed to form a hairpin that can ligate both top and bottom strands at the respective DNA restriction site [22]. In addition, Cap2 contains two centrally-located uracils. Following the single-step ligation of the caps at the two DNA ends, a treatment with uracil glycosylase generates abasic sites at the center of Cap2. During the heating step of the subsequent PCR reaction a strand break is expected to form via beta elimination at the abasic sites, which allows the hairpin to obtain a structure that can be PCR-amplified. To demonstrate the application, an 85 bp p53 sequence flanked by Taq1 and Nla-III restriction sites was generated following a double digestion of the DNA with the two enzymes, and the scheme in **Figure 2C** was applied. **Figure 2D** depicts amplification of the p53 sequence via hairpin-PCR, using primers overlapping the non-complementary linkers and the p53 sequence itself. No amplification was obtained if only one DNA cap was used or if uracil glycosylase were omitted from the protocol.

A mismatch-binding protein, MutS, was previously used to deplete mismatches caused by PCR errors, in order to improve DNA synthesis fidelity [19]. However, genuine mutations are also converted to mismatches and eliminated in this process, thus there is no benefit to mutation detection. In contrast hairpin PCR converts polymerase errors to mismatches while also retains mutations in the homoduplex DNA, thereby enabling the radical elimination of PCR errors via separation (dHPLC, CDCE) or enzymatic (MutS [19], MutY [23], TDG [24]) procedures. We demonstrated amplification of small (~100 bp) sequences in hairpin formation. However polymerases can displace much longer (>1 kb) DNA stretches during synthesis [25]. Therefore adaptation of hairpin PCR to amplify large genomic fractions in a hairpin form, followed by enzymatic depletion of PCR errors could lead to improved genome-wide mutation screening of minute biopsies. Further applications of hairpin PCR can be envisioned. Molecular beacon approaches [13] require the construction of hairpin-shaped probes that interact with the template sequence during real time PCR. In hairpin PCR, the molecular beacon can be the template sequence itself, thus obviating the need for a specific probe.

Experimental protocol.

Hairpin PCR protocols. Hairpins A-D were synthesized by Oligos Etc. (Oregon, USA). Designated amounts of hairpins B-D were then used in a 25 µl PCR reaction using Titanium Advantage^{R} polymerase (Clontech, Palo Alto, CA) and the primers 5'-GTG AGA GGC TGG AGA GTG CT-3', forward; and 5'-ACG TCG ACT ATC CGG GAA CA-3', reverse. PCR thermo-cycling conditions were: 94°, 30 sec; (94°, 30 sec / 68°, 60 sec) x 25 cycles; 68°, 60 sec; 4°; Hold. The products were then examined via ethidium-stained agarose gel electrophoresis. In addition, using the same thermocycling conditions, quantitative real time PCR in the presence of SYBR Green I dye was also performed in a Cepheid I SmartCycler^{TM} machine. Primers used for hairpin A were 5' TAA ATG TTT AAA CAC GCG GT 3', forward; and 5' TAA ATG TTT AAA CAT GCG GT 3'. To amplify picogram amounts of hairpin D spiked into 100 ng genomic DNA, touchdown PCR was applied: 94°, 30 sec; (94°, 20 sec / 65°, 20 sec / 68°, 20 sec)

x 30 cycles, with annealing temperature decreasing 1° /cycle; (94°, 10 sec / 55°, 20 sec / 68°, 20 sec) x 15 cycles; 68°, 6 min; 4°; Hold.

Conversion of a native DNA sequence to a hairpin.
The primers 5'AGG CCT TCA TGA CTG ATA CCA 3' (forward) and 5' TGA GAT CGA CTG AGA CCC CAA 3' (reverse) were used to amplify from genomic DNA a p53 sequence flanked by Taq I and Nla-III sites at the two ends. Following double digestion of the sequence with Nla-III (37°C, 1h) and Taq I (65°C, 1h) the restricted p53 DNA fragment was ligated to the hairpin-shaped sequences Cap1, 5' (phosphate) – CGA CGG CGC GCC GCC TTA GGT AGC GTT AGG CGC GCC GT 3', ligates Taq I-restricted site; and Cap2, 5' (phosphate) –GGC GCG CCG ATG AAG GCA GCT GAC CCC ATC TCA TUU CTT CTC CCC CTC CTC TGT TGC TCA TCG GCG CGC CCA TG 3', ligates Nla-III restricted sites. Following purification with QIAquick™ centrifugation columns (Qiagen Inc, Valencia, CA) the ligation products were treated with uracil glycosylase (37°C, 30 min) in a PCR tube. Following addition of PCR components and buffer a PCR reaction was carried out using the conditions described for hairpins B-D and primers which overlap the ligated Cap2 and the p53 sequence: 5' ATG AGA TGG GGT CAG CTG CCT TCA TCG GCG CGC CCA TGA TTT 3' (forward); and CTT CTC CCC CTC CTC TGT TGC TCA TCG GCG CGC C 3' (reverse).

Figure Legends.

Figure 1. 1A. Scheme for removing PCR errors following amplification of DNA in a hairpin structure. **1B.** Expected structure and sequence of hairpin D, an oligonucleotide encompassing both top and bottom strands of p53 exon 9. Sequences of additional hairpins constructed were: Hairpin A, which is a regular hairpin lacking the non-complementary ends, 5'CAT GAT TTA AAT GTT TAA ACA CGC GGT GGA CTT AAT TAA CTA GTG CCT TAG GTA GCG TGA AAG TTA ATT AAG TCA CCG CAT GTT TAA ACA TTT AAA T 3'; Hairpin B, containing non-complementary ends, 5' ACC GAC GTC GAC TAT CCG GGA ACA CAT GAT TTA AAT GTT TAA ACA CGC GGT GGA CTT AAT TAA CTA GTG CCT TAG GTA GCG TGA AAG TTA ATT AAG TCA CCG CAT GTT TAA ACA TTT AAA TGT ACA GCA CTC TCC AGC CTC TCA CCG CA 3'; Hairpin C, containing non-complementary ends, 5' ACC GAC GTC GAC TAT CCG GGA ACA CAA GAT TTA AAT GTT TAA ACA CGC GGT GAC TTA ACA GGC GCG CCT TAA CTA GTG CCT TAG GTA GCG TGA AAG TTA AGG CGC GCC TGT TAA GTC ACC GCG TGT TTA AAC ATT TAA ATC TTG AGC ACT CTC CAG CCT CTC ACC GCA 3'; Hairpin E, containing non-complementary ends, 5' ACC GAC GTC GAC TAT CCG GGA ACA GAT CCA TGC ACT GCC CAA CAA CAC CAG CTC CTC TCC CCA GCC AAA GAA GAA ACC ACT GGA TGG AGA ATA TTT CGA CCC TTC AGA AAA CTG AAG GGT CGA AAT ATT CTC CAT CCA GTG GTT TCT TCT TTG GCT GGG GAG AGG AGC TGG TGT TGT TGG GCA GTG CAT GGA TCA GCA CTC TCC AGC CTC TCA CCG CA 3'. **1C.** Hairpin-PCR. Lanes 1-5, PCR product of hairpins A,B,C,E, and D, respectively. Lanes 6 and 7, amplification of hairpin D with only forward or only reverse primer. **1D.** Amplification of hairpin C using Advantage Titanium® (lane 1), Pfu Turbo® (lane 3) or Advantage HF2® (lane 5) polymerases respectively. Lanes 2, 4 and 6 are water-controls (no template) in each case. **1E.** Hairpin PCR (lanes 1 and 2, in duplicate), followed by denaturation and rapid cooling of the product (lanes 3 and 4, in duplicate). **1F.** Hairpin D amplified with primers that bind to the non-complementary ends, and either not extending (**lane 1**) or extending 9 bases into the hairpin sequence (**lane 2**). **1G.** Quantitative real time PCR of

hairpin D. Curves 1-4, starting material of 1 ng, 100 pg, 10 pg and 1 pg respectively. **1H**. Spiking of p53 exon 9-containing hairpin D into 100 ng p53-negative whole genome, followed by hairpin PCR using Advantage Titanium® polymerase. Spiking of 0.01 pg hairpin D corresponds to adding a single p53 exon 9 allele in the genome. Lanes 1-6, hairpin D addition of 0, 0.1, 1, 10, 100, 1000 pg respectively. **1I**: Similar to 1H, but using Advantage HF2® polymerase. Lanes 1-5, hairpin D addition of 0, 0.01, 0.1, 1, 10 pg. **1J**. dHPLC – based separation of 1:1 mixtures of homoduplex and heteroduplex PCR-amplified hairpins. The threshold of the fraction collector is set on the trailing (slowest) portion of the homoduplex.

Figure 2. Conversion of a sequence to a hairpin. **2A**. Procedure used to convert a native DNA sequence, flanked by two different restriction sites, into a hairpin with non-complementary ends that can be amplified. The hairpin-shaped oligonucleotides Cap1 and Cap2 are ligated to the 5' and 3' of both sequence ends. During PCR, primers extending into the sequence are used, to confer sequence specificity. **2B**. Conversion of a native p53 sequence to a hairpin, followed by hairpin-PCR. Lane 1: omission of Cap1 from the ligation reaction. Lane 2: Amplification of an 85 bp p53 sequence using the scheme in 2A. Lane 3. Omission of uracil glycosylase from the procedure. Lane 4. Omission of one primer from the PCR reaction.

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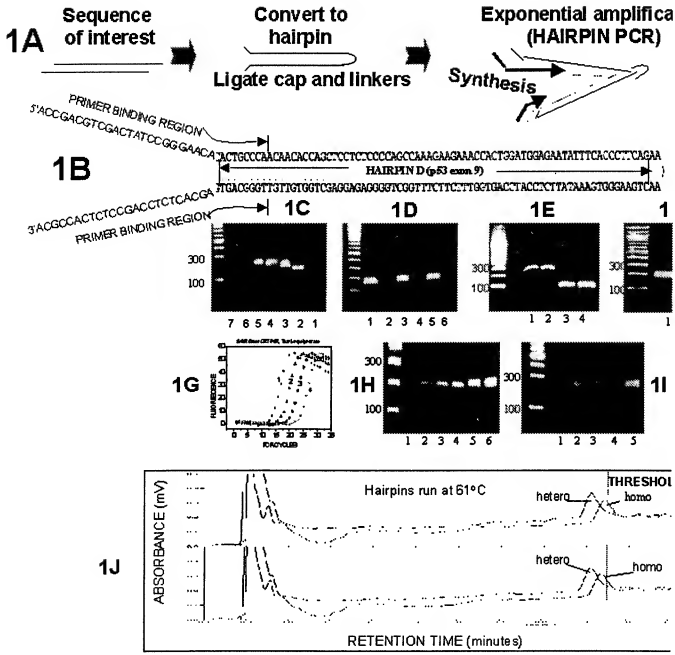
1A
Sequence of interest
Convert to hairpin
Ligate cap and linkers
Exponential amplifica
(HAIRPIN PCR)
Synthesis
1B
PRIMER BINDING REGION
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ACTGCCCAACAACACCAGCTCCTCCCCCAGCCAAAGAAGAAACCACTGGATGGAGAATATTTCACCCTTCAGAA
HAIRPIN D (p53 exon 9)
TTGACGGGTTGTTGTGGTCGAGGAGAGGGGTCGGTTTCTTCTTGGTGACCTACCCTTATAAAGTGGGAAGTCAA
3'ACGCCACTCTCCGACCTCTCACGA
PRIMER BINDING REGION
1C
1D
1E
1
300
100
7 6 5 4 3 2 1
1 2 3 4 5 6
1 2 3 4
1
1G
1H
1I
CYCLES
1 2 3 4 5 6
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ABSORBANCE (mV)
Hairpins run at 61°C
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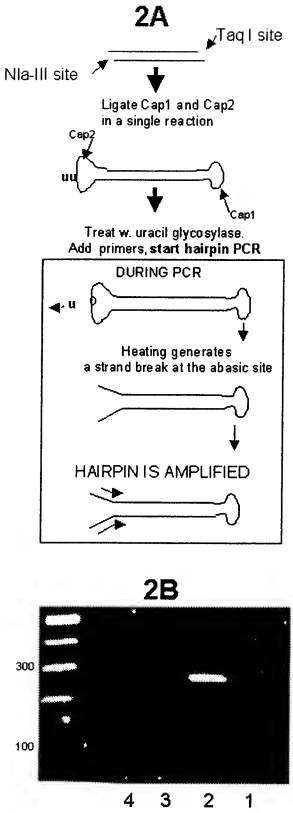
RETENTION TIME (minutes)


2A
Taq I site
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Ligate Cap1 and Cap2
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Treat w. uracil glycosylase.
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HAIRPIN IS AMPLIFIED
2B
300
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4 3 2 1